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Optimizing Parameters for WII STED Imaging
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Stimulated emission depletion (STED) nanoscopy is one of the most important recent innovations in biological imaging. This technology enables noninvasive study of biological specimens with nanometer resolution.
The most powerful versions of STED nanoscopes rely on pairs of synchronized pulsed laser beams. The excitation beam is focused into the sample, producing an ordinary diffraction limited spot of excited molecules. The red shifted and doughnut shaped depletion beam follows immediately the excitation, quenching the fluorescence to the ground state by stimulated emission.
Thus the diffraction barrier can be overcome narrowing the fluorescence spot by saturating the stimulated emission transition. Although the STED concept can be formulated in a simple equation[1][2], the realization of such a nanoscope needs several experimental precautions to exploit the maximum resolution achievable. We will report on detailed experimental studies focusing on the performance of the microscope in dependence on the arrival times of the corresponding excitation and STED pulses as well as the influence of the polarization of the STED beam to the quality of the donut. The latter has a direct influence to the final image quality and is of great importance.
Moreover we will show the effects and the enhancement that can be achieved by applying time gating detection.
[1] Hell, S. W. 2004. Strategy for far-field optical imaging and writing without diffraction limit. Physics Letters A 326:140-145.
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Quantitative Characterization of Dyes Embedded in Nanoparticles by Single-Molecule Fluorescence
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Many multi-component nanoparticles are being designed for multi-modality imaging or theragnostics (diagnostics plus therapy). Dyes are often embedded into multi-modal nanoparticles for fluorescence imaging. Quantitative characterization of dyes in the nanoparticles is difficult because dye properties are often changed compared to free dyes. Accurate determination of the number of dyes in each nanoparticle, the brightness and the photostability of dyes, and the number of nanoparticles without any dye is challenging with ensemble methods. Exploiting the photobleaching property of dyes, we present a Single Particle Observation Technique (SPOT) based on total internal reflection fluorescence microscopy (TIRFM) to characterize dyes embedded in silica-coated iron oxide nanoparticles. Using this high throughput method, we can accurately determine the aforementioned parameters using very little sample. We have developed a robust statistical analysis of the number distribution of the dyes to determine the percentage of nanoparticles without any dye (labeling efficiency) and independently verified the statistical estimate of the labeling efficiency using a combination of phase contrast and fluorescence imaging. Our approach is readily applicable to systems such as dye-labeled proteins and DNA even when the labeling efficiency is not 100%.

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Evaluation of Fluorophores for Optimal Performance in Localization-Based Super-Resolution Imaging
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A variety of approaches have recently been developed to surpass the diffraction-limited resolution of fluorescence microscopy. One such approach relies on the sequential activation and nanoscale localization of single fluorophores, where the high-precision localizations of individual molecules can be used to construct a sub-diffraction-limit image with dramatically improved spatial resolutions. Photoswitchable fluorescent probes have become an essential component in localization-based super-resolution imaging. The properties of these probes, such as the photons per switching event, on/off duty cycle, photostability, and number of switching cycles, determine a probe's effectiveness for creating high resolution images. Yet a quantitative characterization of these properties for most fluorophores is lacking, preventing an effective and systematic choice of optimal fluorophores and imaging conditions for attaining high quality super-resolution images. To address this need, we systematically characterize the switching properties of tens of organic dyes and demonstrate how these properties directly impact the quality of a super-resolution image. Our analysis provides a set of guidelines to be followed for evaluating fluorophores and is a resource for probe selection for interested practitioners of single molecule localization-based super-resolution imaging. In addition to our quantitative characterization, we also identify several new dyes suitable for this imaging modality. Using the highest performing dyes from our screen in each of four distinct spectral ranges, we demonstrate low crosstalk, four-color super-resolution imaging and apply these new imaging capabilities to study various cellular structures.

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A Custom-Built Fast Scanning STED Microscope with a Large Field of View
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In classical microscopy the resolution is limited to ~200-300 nm due to Abbe's diffraction limit. In the past two decades several techniques emerged to overcome this limit, among which the STimulated Emission Depletion (STED) microscopy attracted wide interest (Hell and Wichmann, *Opt. Lett.* 19:780-782, 1994). We built a resonant scanning STED microscope, which achieves a lateral resolution in biological samples of ~40 nm in a large scanning area of ~40x40 µm acquired at ~10 nm/pixel.
In STED microscopy, two beams are required: one excites the sample as in classical confocal microscopy, while the other one, with a donut-shape profile, de-excites the fluorophores by stimulated emission, which avoids the fluorescence emission. Only the tiny volume located at the center of the donut will emit fluorescence. The size of this emitting spot defines the resolution of the STED images and strongly depends on the quality and the intensity of the STED beam. To be able to acquire images of ~4000x4000 points at a speed of 16,000 lines per second, an 8 KHz resonant scanning mirror synchronized with a 96 Mhz frame grabber were used. Channels, receptors and signaling molecules in isolated cardiac mitochondria, cardiomyocytes, smooth muscle cells and epithelial cells were immunolabeled after fixation and/or *in vivo* with specific antibodies and Atto-647N as the secondary antibody. At fast scanning speed the photobleaching of samples was considerably reduced, allowing repeated imaging of the same area and the acquisition of a stack of planes for 3D reconstruction. With this microscope, we have uncovered a discrete distribution of proteins within a single mitochondrion, and that plasma membrane proteins are distributed in discernable clusters of different sizes. Supported by NIH and AHA.

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Superresolution STED Imaging Reveals a Periodic Punctate Pattern of Adenylyl Cyclase Type III on Primary Cilia
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Primary cilia are organelles serving essential sensory and signaling functions in nearly all mammalian cell types. They serve as a center of complex signaling, involved in cAMP, Wnt and Shh signaling pathways. Adenylyl cyclases type III (ACIII) is a primary cilia marker involved in cAMP signaling, playing important roles in regulating ciliogenesis and sensory function. Despite its importance, detailed ACIII localization and their interactions with other proteins remain unclear due to the limited resolution of conventional microscopy. To determine the morphological characteristics of ACIII in primary cilia, we conducted superresolution imaging of immunostained ACIII in fibroblasts and neurons using stimulated emission depletion (STED) microscopy, which allows us to resolve the localization of ACIII achieving a resolution of 50 nm. In contrast to the previous understanding that ACIII distributes uniformly along a primary cilium, our STED images revealed that ACIII formed a periodic punctate pattern with a roughly equal spacing between groups of puncta. These puncta occupied less than 50% of the area, with the size of 137 ± 20 nm in the axial direction along the primary cilia. The spacing between puncta was 250 ± 67 nm. Some primary cilia even showed two rows of periodic puncta along the axial direction, with a tilted angle of about 12° to 35° between the two rows. The spacing between the two rows was 195 ± 19 nm. In some cells, ACIII was only localized in the basal body, where the periodic punctate pattern was absent. Based on our superresolution studies, we concluded that ACIII can be transported into a primary cilium, but would only occupy regions approximately equally spaced along the cilium.